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Janett, F ; Gerig, T ; Tschuor, A C ; Amatayakul-Chantler, S ; Walker, J ; Howard, R ; Piechotta, M ; Bollwein, H ; Hartnack, S ; Thun, R

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F. Janett [a, *], T. Gerig [a], A.C. Tschuor [b], S. Amatayakul-Chantler [c], J. Walker [c], R. Howard [c], M. Piechotta [d], H. Bollwein [a], S. Hartnack [e], R. Thun [a]

[a] *Clinic of Reproductive Medicine, Vetsuisse-Faculty University of Zürich, Winterthurerstrasse 260, 8057 Zürich, Switzerland*

[b] *Cattle practice Tschuor and Gartmann, Via Gravas 7, 7130 Ilanz, Switzerland*

[c] *Pfizer VMRD, 45 Poplar Road, Parkville, 3052 Melbourne, VICTORIA, Australia*

[d] *Clinic for Cattle, University of Veterinary Medicine Hannover Foundation, Bischofsholer Damm 15, 30173 Hannover, Germany*

[e] *Section of Epidemiology, Vetsuisse-Faculty University of Zürich, Winterthurerstrasse 270, 8057 Zürich, Switzerland*

Abstract

The aim of this study was to evaluate the effect of immunization against gonadotropin-releasing factor (GnRF) with Bopriva® (Pfizer Animal Health, Parkville, Australia) in prepubertal bull calves. For the study, 6 calves were vaccinated at the age of 3 and 6 weeks with 1 mL Bopriva®, and 6 animals served as matched controls. Concentrations of GnRF antibodies, testosterone and LH were determined in serum samples out to 30 weeks after the first immunization. Body weight and scrotal circumference were measured for 59 weeks. At slaughter, 65 weeks after the first immunization, the quality of epididymal sperm was evaluated. The results showed that vaccination against GnRF influenced ($P < 0.05$) anti-GnRF titer, LH and testosterone concentrations as well as scrotal circumference. Antibody titers significantly ($P < 0.05$) increased after the booster vaccination and reached peak values 2 weeks later. Compared to control animals, inhibition ($P < 0.05$) of the prepubertal LH

secretion was observed in vaccinated calves at weeks 10 and 12 to 14 after the first vaccination. In vaccinated calves testosterone concentrations decreased after the booster injection to values below 0.5 ng/mL serum and remained for at least 22 weeks at this low level. Animals vaccinated with Bopriva® showed a delay in testes growth and smaller scrotal circumference. Puberty occurred at the age between 46 and 55 weeks in vaccinated and between 38 and 52 weeks in control animals and body weight gain was similar in both groups. All vaccinated bulls attained spermatogenic capacity at slaughter when they were 68 weeks old.

Keywords:

Calf

Castration

GnRF

GnRH

Vaccine

Puberty

1. Introduction

Immunological methods represent animal-friendly alternatives to physical castration in animals. Immunization against sex hormones, LH receptors, sperm antigens or zona pellucida proteins has been described (Fayrer-Hosken, 2008). In the male as in the female, GnRF (gonadotropin-releasing factor) plays a central role in the regulation of sexual function. After immunization against GnRF, antibodies neutralize the endogenous GnRF resulting in the suppression of secretion of the gonadotropins LH and FSH in the anterior pituitary. The effectiveness of vaccination against GnRF could be demonstrated in numerous studies in

cattle (Aissat et al., 2002; Cook et al., 2000; D'Occhio et al., 2001; Finnerty et al., 1998; Geary et al., 2006; Hoskinson et al., 1990; Jago et al., 1997; Jeffcoate et al., 1982; Neeson and Colson, 2004; Stevens et al., 2005; Theubet et al., 2010), sheep (Brown et al., 1994, 1995; Ferro et al., 2004; Janett et al., 2003, 2009a; Jeffcoate et al., 1982; Ülker et al., 2005), pig (Bonneau et al., 1994; Clarke et al., 2008; Claus et al., 2007; Dunshea et al., 2001; Fuchs et al., 2009; Jaros et al., 2005; Zamaratskaia et al., 2008), horse (Dalin et al., 2002; Elhay et al., 2007; Imboden et al., 2006; Janett et al., 2009b; Schanbacher and Pratt, 1985; Turkstra et al., 2005) and in wild animals (Curtis et al., 2002; Miller et al., 2000, 2004).

Regarding reversibility of immunization against GnRF, it has been known that the effects on fertility are individual and may last for a prolonged time (Brown et al., 1994, 1995; Clarke et al., 1998; Janett et al., 2003). Ram lambs immunized twice at an age of 3 to 4 weeks and 13 to 14 weeks against GnRF showed a decreased testicular size when they reached 2 years of age (Brown et al., 1994). In another study (Janett et al., 2003) one out of 10 peripubertal vaccinated ram lambs had low testicular size and blood testosterone concentrations for more than 1 year after the booster vaccination. Also in pre- and peripubertal immunized female lambs, 60% displayed no estrus and small uteri as well as ovaries at 2 years of age (Brown et al., 1995). These studies clearly demonstrate that inhibition of GnRF secretion in juvenile animals can hamper fertility in adulthood. In bull calves, blood concentrations of gonadotropins increase long before the animal reaches puberty. It has been shown that LH starts to rise from 6 weeks of age reaching peak values between 12 to 16 weeks and subsequently drops to basal concentrations at week 25 of life remaining low until attainment of puberty (Amann and Walker, 1983; Aravindakshan et al., 2000; Bagu et al., 2006; Evans et al., 1996; Lacroix and Pelletier, 1979; McCarthy et al., 1979; Rawlings and Evans, 1995; Rawlings et al., 1978, 2008; Rodriguez and Wise, 1989). The early rise in gonadotropin secretion seems to be essential for normal testicular development and reproductive maturation in bull calves (Evans et al., 1995; Rawlings et al., 2008). Inhibition of GnRF secretion by

application of a long-acting GnRF agonist in 6 to 18 week old calves has been shown to led to a marked delay in testicular development (Chandolia et al., 1997). Similarly sustained inhibition of GnRF secretion by vaccination against GnRF early in life may also suppress the prepubertal rise in GnRF and offer a true alternative to castration in bull calves. Results from a pilot project (Theubet et al., 2010) using the anti-GnRF vaccine Bopriva®, (Pfizer Animal Health, Parkville, Australia) designed and marketed specifically for cattle, did demonstrate that pubertal vaccination suppressed testicular growth and testosterone secretion for a duration of at least 10 weeks. Based on the assumption that the prepubertal rise in gonadotropins is required for normal testicular development, the aim of the present investigation was to evaluate the effects of vaccination with Bopriva® early in life on LH secretion as well as on testicular growth and function in the bull calf.

2. Material and methods

2.1. Experimental design

For the experiments 12 Holstein Friesian bull calves, all born within a week of each other, were purchased in the first week of life from 9 different farms. For the first 14 weeks of age they were fed skim powder-based milk replacer with free access to water, hay, straw and minerals. Until 8 months of age (period 1), the animals were housed together in a group corral on straw with regular access to pasture. From the age of 8 months until slaughter at 15 months (period 2) the bulls were kept in a pen on straw and a high-energy finishing diet was provided. At the beginning of the experiment 6 animals were randomly allocated to a treatment group (A-F) and 6 calves served as controls (G-L). At the age of 3 and 6 weeks the animals of the treatment group were vaccinated subcutaneously with 1 mL Bopriva® (400 µg GnRF-protein conjugate, Pfizer Animal Health, Parkville, Australia) on the right side of the neck. Control calves received the same amount of saline solution. Adverse effects were monitored daily for

1 week after treatment with particular regard to body temperature as well as swelling and pain at the injection site. Thereafter, weekly examinations were carried out until all reactions had subsided.

To evaluate treatment effects, animals were examined weekly for 8 months in period 1 and every second week for further 7 months in period 2. At each occasion calves were weighed and blood was collected by venipuncture of the Vena jugularis externa dextra (period 1) or Vena coccygealis ventralis (period 2) using vacutainers (9 mL Z Serum Clot Activator® Vacuette®, Greiner Bio-One GmbH, Kremsmünster, Austria). The blood samples were allowed to clot during 2 h at room temperature and after centrifugation (4000 x *g*, 10 min) serum was frozen (-18 °C) until analysis. From 8 weeks of treatment scrotal circumference was determined (ReliaBull®, Lane Manufacturing Inc., Denver, USA) in all animals. All animal experimentation was performed following approval from the local Animal Ethics Committee.

2.2. Hormone analysis and GnRF antibody titers

GnRF antibody titers

Serum anti-GnRF antibody titers were determined by dissociation enhanced lanthanide fluorescence immunoassay (DELFIA) (Perkin Elmer Pty Ltd, Glen Waverly, Australia). Briefly, 384-well streptavidin coated plates (Perkin Elmer Pty Ltd, Glen Waverly, Australia) were coated for 1 h at room temperature with 1 µg/mL biotinylated GnRF peptide in DELFIA buffer (50mM Tris-HCl, 0.9 % NaCl, 0.05 % Tween 20, 20 µM EDTA, 0.2 % ovalbumin). Plates were washed and then incubated with serial dilutions of test cattle serum for 1 h at room temperature. Unbound serum and antibodies were removed by washing, and bound antibody was detected by incubating plates for a further 1 h with europium labeled protein G (Perkin Elmer Pty Ltd, Glen Waverly, Australia). After washing off excess europium labeled

protein G, DELFIA Enhancement Solution (Perkin Elmer Pty Ltd, Glen Waverly, Australia) was added to all wells. After 10 min plates were excited at 340 nm and emission readings at 615 nm were measured. Serial dilutions of a standard with a reciprocal titer of 1/409,600 served as a reference for unknown samples. Reciprocal titer was determined as the highest serum dilution at which the 615 nm fluorescence was two standard deviations higher than the negative control. Non-vaccinated cattle serum served as a negative control.

Testosterone concentrations

Testosterone was determined by enzyme-amplified sensitivity immunoassay (TESTO-EASIA, BioSource Europe S.A, Nivelles, Belgium). The detection limit of the assay was 0.01 ng/mL. All samples were analyzed using a competitive binding assay where a fixed amount of testosterone labeled with horseradish peroxidase (HRP) competes with unlabelled testosterone present in calibrators, controls and samples. Cross reactivity with estrogens and progesterone was 0.023 % and 0.035 %, respectively, with androstenedione 0.76 % and 5-α-dihydrotestosterone 0.61 %. The intra- and inter-assay coefficients of variance were 6.3 % and 8.3 %, respectively.

LH concentrations

LH measurements were performed using enzyme-linked immunosorbent assay (ELISA) (LH-DETECT®, ReproPharm, Nouzilly, France) with a sensitivity of 0.01 ng LH/mL. The intra- and inter-assay coefficients of variance were 5.9 % and 8.4 %, respectively.

2.3. Testicular weight and sperm quality

From the testes recovered after slaughter, the cauda epididymis including the proximal vas deferens was separated from each testis and paired testes weight determined. For the collection of epididymal sperm the ductus deferens and the cauda epididymis were flushed in a retrograde direction with 10 mL bull semen extender (AndroMed®, Minitüb GmbH, Tiefenbach, Germany). Harvested semen from both caudae epididymidae of each bull was extended and total sperm count as well as sperm motility were determined with a sperm analyzer (CASA) (Hamilton Thorne IVOS, Version 14, Beverly, MA, USA) using standardized settings for bull semen (Table 1). For the measurements 5 µL of the diluted semen were pipetted into a 20 µm standard count analysis chamber (Art. Nr. SC 20-01-C, Leja, Nieuw-Vennep, Netherlands) and a minimum of 15 fields evaluated by CASA. For morphological examination, one drop of semen was fixed in 2 mL buffered formol saline solution (Na2HPO4 4.93 g, KH2PO4 2.54 g, 38 % formaldehyde 125 mL, NaCl 5.41 g, distilled water qs 1000 mL) and smears prepared. At least 200 spermatozoa were subsequently evaluated by phase contrast microscopy (Olympus BX50, UplanF1 100x/1.30) and abnormal spermatozoa classified in major (acrosome defects, nuclear vacuoles, abnormal heads, loose abnormal heads, abnormal midpieces, proximal droplets) and minor (loose normal heads, abnormal tails, distal droplets) defects (Blom, 1973).

Table 1

CASA settings for bull semen.

Parameter	Setting
Frames aquired	30
Frame rate	60 Hz
Minimum contrast	80
Minimum cell size	5 pix
Minimum static contrast	15
Straightness (STR), threshold	70 %
VAP cutoff	30.0 μm/s
Prog. min. VAP	50.0 μm/s
VSL cutoff	15.0 μm/s
Cell size	5 pix
Cell intensity	70
Static head size	0.1 to 3.4
Static head intensity	0.3 to 1.7
Static elongation	8 to 97
Slow cells motile	NO
Magnification	1.89
Video frequency	60
Bright field	NO
LED illumination intensity	2330
IDENT Illumination intensity	3500
Temperature, set	37.5 °C
Chamber depth	20 μm
Chamber position	4.0 mm
Chamber position B	19.0 mm
Chamber position C	0 mm
Chamber position D	0 mm
Chamber type	Leja
Field selection mode	AUTO
IDENT fluorescent option	OFF
Integrating time	1 frame

2.4. Statistical analysis

The data were analyzed using R: A Language and Environment for Statistical Computing (R Foundation for Statistical Computing, Vienna, Austria) version 2.14.0 and the software package nlme (Pinheiro et al., 2011). To assess a potential effect of immunization on the outcome variables anti-GnRF titer, LH and testosterone concentrations, scrotal circumference and body weight, linear mixed effects models were used. Due to the data with measurements over time and potential clustering within-animal, calf was treated as random effect. Different

models were fitted with different explanatory variables (group, time and an interaction between group and time), different random structures with and without time as a random slope and variance structures allowing for different variances between groups. Model selection was used to assess the degree of statistical support for either constant variance or heterogeneous variances between groups and to decide which of the explanatory variables should be included in the final model. Akaike's information criterion (AIC) was the model selection metric with lower AIC values indicating a better model fit. Model validation was done by checking visually the residuals for homogeneity (plotting residuals versus fitted values), normality (histogram of residuals) and independence (residuals versus explanatory variables). Attainment of puberty was defined as the age when bulls reached the critical scrotal circumference of 28 cm (Lunstra et al., 1978; Wolf et al., 1965). Pairwise comparisons were performed after Bonferroni adjustment in parametric and Mann-Whitney test in non-parametric distributed data, respectively. Values were considered to be statistical significant at $P < 0.05$.

3. Results

3.1. Adverse effects

All calves treated with Bopriva® showed no signs of apathy but 1-2 days after the first and second immunization, an individual rise in body temperature to peak values of 40.7 °C was observed. A slight non-painful swelling at the injection site developed in all vaccinated animals and spontaneously subsided within 2-4 weeks. In control animals, body temperature varied between 37.9 °C and 40.1 °C and no adverse effects could be seen.

3.2. Effects of vaccination

Effects of group, time of examination and interaction of group and time of examination on evaluated parameters are shown in Table 2. It is apparent that the time of examination significantly ($P < 0.05$) influenced all parameters. The group and the interaction of group and time of examination significantly influenced all parameters with exception of body weight.

Table 2
Effects of group, time of examination and interaction of group and time on evaluated parameters.

Parameter	Group *P*	Time *P*	Interaction *P*
Anti-GnRF titer	<0.0001	<0.0001	<0.0001
LH concentration	0.0381	<0.0001	0.0108
Testosterone concentration	0.0124	<0.0001	<0.0001
Scrotal circumference	0.0198	<0.0001	<0.0001
Body weight	n.s.	<0.0001	n.s.

n.s.: not significant ($P > 0.05$)

Anti-GnRF titer

Mean (± SEM) serum anti-GnRF antibody titer in bull calves with and without Bopriva® for 30 weeks after first immunization are shown in Fig. 1a. In vaccinated animals antibody titer began to rise slightly 2 weeks after the first injection and reached peak values of 499,192 ± 100,983 units 2 weeks after the booster immunization. Thereafter, the titer slowly decreased and reached basal values 28 weeks after first vaccination. In control animals titers remained very low. Significant differences between groups were apparent in weeks 2-24 and 27.

Maximum titers showed large individual variations and ranged between 868,025 units (calf F) and 138,273 units (calf C). Initial values were reached individually in weeks 21 (calf C), 25 (calves A, E), 26 (calf D) and 28 (calves B, F).

LH

Mean (± SEM) serum LH concentrations in bull calves with and without Bopriva® for 30 weeks after first immunization are shown in Fig. 1b. Before the booster injection the values fluctuated at a low level between 0.14 ± 0.03 ng/mL and 0.24 ± 0.02 ng/mL in vaccinated calves and between 0.13 ± 0.01 ng/mL and 0.19 ± 0.03 ng/mL in control animals. Thereafter and until week 22 the LH concentration in vaccinated calves ranged between 0.01 ± 0.00 ng/mL (weeks 12, 13, 14) and 0.42 ± 0.02 ng/mL (week 22) whereas in control animals the values ranged between 0.13 ± 0.03 ng/mL (week 15) and 0.57 ± 0. 05 ng/mL (week 22). From week 23 to week 30 LH continuously increased in both groups and varied between 0.42 ± 0.02 ng/mL and 0.59 ± 0.01 ng/mL in treated calves and between 0.42 ± 0.01 ng/mL and 0.58 ± 0.03 ng/mL in controls. Significant differences between groups were identified at weeks 10, 12-14, and 22.

Testosterone

Mean (± SEM) serum testosterone concentrations in bull calves with and without Bopriva® were followed for 30 weeks after first immunization as shown in Fig. 1c. In vaccinated animals the values were always < 0.5 ng/mL during weeks 0-24 and continuously increased thereafter. Testosterone concentrations in control animals were > 0.5 ng/mL except at weeks 0-2, 4 and 15. Significant differences between groups were noted for weeks 5-12, 14-17, 21 and 25.

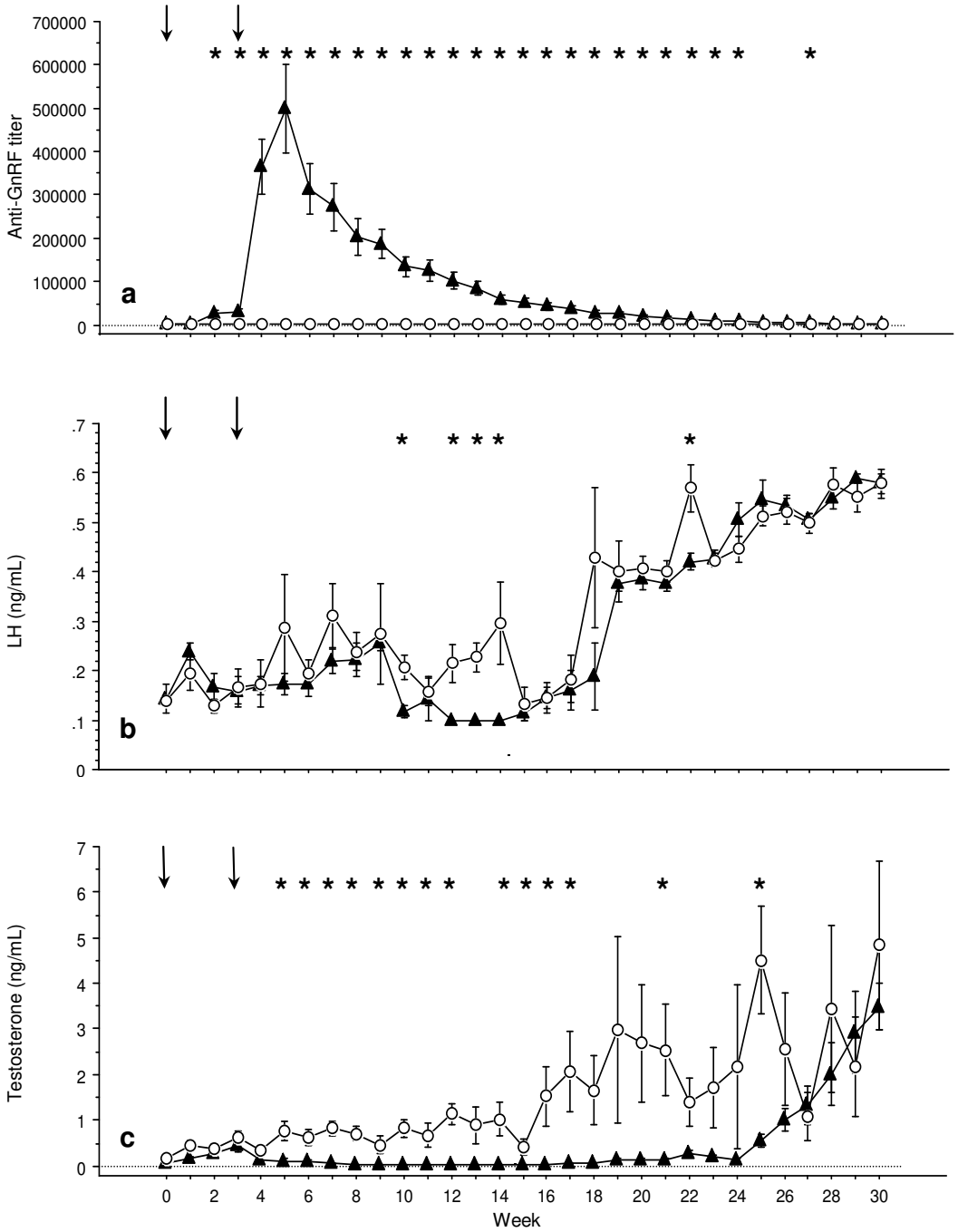

Fig. 1. Mean (± SEM) anti-GnRF titer (a), serum LH (b) and testosterone (c) concentrations in bull calves with (▲, n=6) and without (○, n=6) Bopriva®. Arrows indicate injections. *Significant ($P < 0.05$) difference between groups.

Scrotal circumference and body weight

Mean (± SEM) scrotal circumference in bull calves with and without Bopriva® for weeks 8 to 59 after first immunization are shown in Fig. 2a. In both groups the course of scrotal circumference followed the pattern of a sigmoid curve. During an initial phase of low testicular growth during weeks 8-21 scrotal circumference ranged between 13.7 ± 0.4 cm and 14.8 ± 0.2 cm in vaccinated calves and between 14.6 ± 0.5 cm and 17.2 ± 0.7 cm in control animals. Thereafter testes grew faster until week 49 when scrotal circumference reached 29.9 ± 1.0 cm in calves with and 33.2 ± 1.2 cm in calves without Bopriva®. From week 50 growth slowed in both groups and values of 33.5 ± 0.8 cm and 35.8 ± 1.2 cm were reached by week 59 in vaccinated and control animals, respectively. Significant differences between groups were present for weeks 12, 17-18, 20-37, 43 and 45.

Mean (± SEM) body weight in bull calves with and without Bopriva® during 59 weeks after first immunization are shown in Fig. 2b. The weight gain was similar in both groups and body weight increased from 50.8 ± 1.4 kg and 47.2 ± 3.4 kg in week 0 to 535.2 ± 18.3 kg and 507.3 ± 27.8 kg in week 59 in vaccinated and in control animals, respectively. At slaughter, at an age of 68 weeks, body weight of bulls with and without Bopriva® was 591 ± 21 kg (range 490-627 kg) and 562 ± 27 kg (range 442-631 kg), respectively. Differences between groups were not significant ($P > 0.05$).

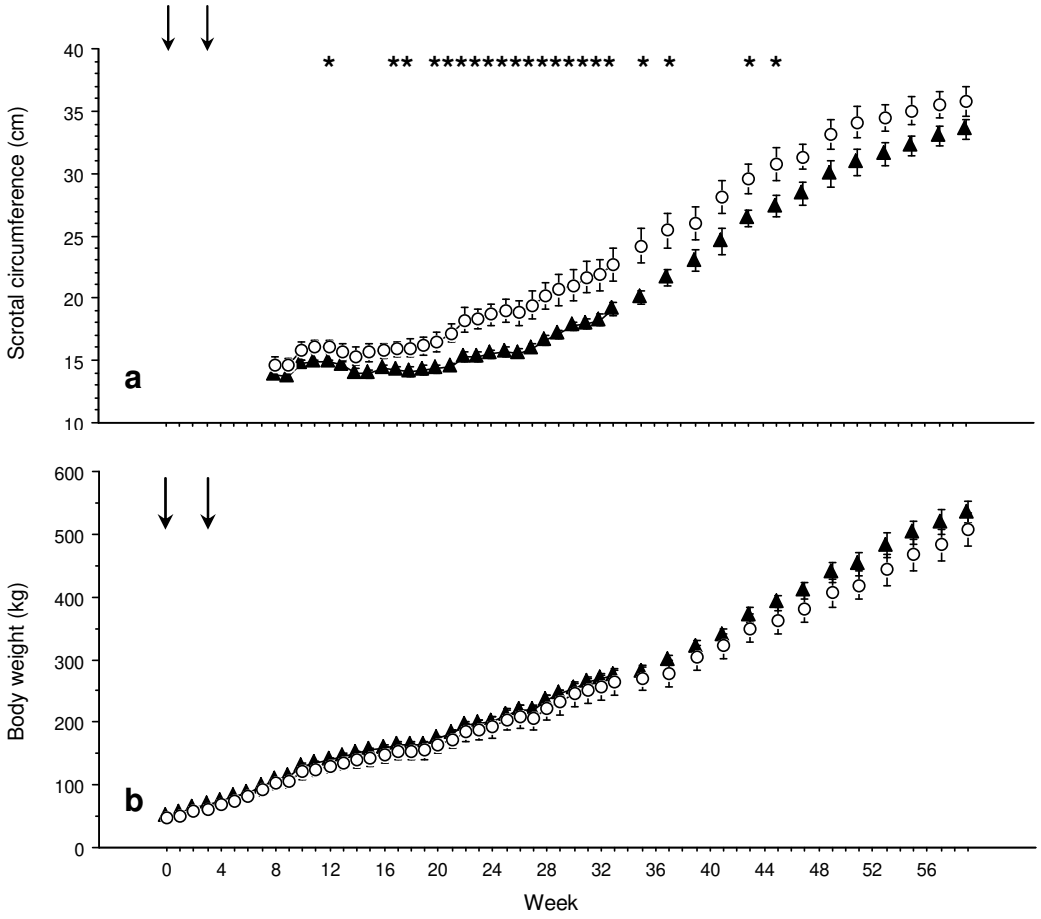

Fig. 2. Mean (± SEM) scrotal circumference (a) and body weight (b) in bull calves with (▲, n=6) and without (○, n=6) Bopriva®. Arrows indicate injections. *Significant ($P < 0.05$) difference between groups.

Age at puberty, testicular weight and semen quality

Vaccinated calves attained puberty at the age between 46 and 55 weeks (median 50 weeks) and in control animals between 38 and 52 weeks of age (median 47 weeks) as shown in Fig. 3a.

Testicular weight was determined at slaughter at week 65, when the bulls were 68 weeks old as shown in Fig. 3b. Paired testes weight ranged between 481 g and 634 g (median 573 g) in vaccinated and between 508 g and 831 g (median 666 g) in control animals.

Total sperm count of semen harvested from the epididymis at slaughter ranged between 3,748 x 10^6 and 7,676 x 10^6 (median 4,679 x 10^6) in bulls with Bopriva® and between 2,655 x 10^6 and 9,240 x 10^6 (median 5,834 x 10^6) in control bulls (Fig. 3c).

Sperm motility ranged between 26 % and 68 % (median 43 %) in vaccinated and between 12 % and 77 % (median 54 %) in control animals (Fig. 3d). The percentage of sperm with major (Fig. 3e) and specifically acrosome (Fig. 3f) defects ranged between 16 % and 72 % (median 29 %) and between 2 % and 7 % (median 4 %) in vaccinated animals, respectively. In control animals major sperm defects ranged between 12 % and 93 % (median 59 %) and acrosome defects between 1 % and 9% (median 5 %), respectively. No significant ($P > 0.05$) differences in age at puberty, testicular weight and semen characteristics were apparent between groups.

4. Discussion

Results of this study show that compared to control animals vaccination of bull calves with Bopriva® at an age of 3 and 6 weeks suppressed testicular development and endocrine function for more than 22 weeks. General health, suckling and weight gain were not affected by immunization against GnRF. Also, semen quality evaluated at the end of the experiment was similar in bulls with and without Bopriva®. However, a temporary increase in body temperature and a slightly transient swelling at the injection site was observed in all vaccinated animals. The high degree of safety of Bopriva® is mainly due to the fact that the vaccine was developed specifically for cattle and that the adjuvant used is well tolerated in the bovine species.

After the first immunization, all calves showed a low anti-GnRF antibody titer which rapidly increased following the booster injection. This confirms the findings of Chase et al. (2008), that 3 to 6 weeks old calves are able to produce a reliable immune response. Titers determined in this study were even higher and more sustained than those measured in a previous

investigation using Bopriva® in pubertal bulls (Theubet et al., 2010). One explanation for the inconsistent titer reaction could be a more efficient antigen presentation in the lymph nodes as in the present study both immunizations were applied on the same site of the neck and not on opposite sites (Theubet et al., 2010).

LH concentration was influenced by vaccination against GnRF and significantly lower mean values were measured in vaccinated compared to control calves at weeks 10 and 12 to 14 after the first injection. Moreover, fluctuations in LH were less pronounced in animals with Bopriva® indicating a different LH secretion pattern caused by vaccination against GnRF. Control animals showed a continuous increase of LH to levels of 0.3 ng/mL serum. These values are much lower than concentrations of about 1.6 ng/mL reported in previous studies (Evans et al., 1993; Bagu et al., 2006), the reason being most likely due the method of LH analysis used, breed differences and most important the frequency of blood sampling. In the experiments mentioned above blood was collected at short intervals of 10-12 min during several hours and the basal, amplitude and mean concentrations of LH determined. This approach allows detecting increases in LH pulse frequency which is mainly responsible for the prepubertal gonadotropin rise (Evans et al., 1993) more reliably than by single weekly blood collections as in the present study. After week 17 of the experiment (week 20 of life) LH increased rapidly and then more slowly up to values of approximately 0.6 ng/mL in both control and vaccinated calves. Such a rise in LH values after the 20th week of life has not been reported in previous studies (Aravindakshan et al., 2000; Bagu et al., 2006; Chandolia et al., 1997; Evans et al., 1993). A possible cause for the different findings may be breed influence, as our experiment was performed with Holstein calves whereas Hereford (Chandolia et al., 1997; Evans et al., 1993) or crossbreeds of Hereford and Charolais (Aravindakshan et al., 2000; Bagu et al., 2006) were used in the previous studies. Moreover, it has been known that diet and weight gain may also influence LH secretion and the attainment of puberty (Barth et al., 2008; Brito et al., 2007a, 2007b).

Testosterone secretion was suppressed concomitant with the rise of anti-GnRF titers after booster vaccination. The duration of testosterone inhibition lasted for at least 22 weeks and concentrations started to increase individually when titers reached baseline values. Surprisingly, a delay of 6 weeks between the resumptions of LH and testosterone secretion was observed. The persistently low testosterone values despite increased LH concentrations suggest that the immunization effect is not solely based on the neutralization of GnRF but also a weaker responsiveness of testicular receptors to gonadotropins. It might be possible that vaccination leads to delayed formation of LH receptors on Leydig cells as also assumed by elevated testicular temperatures in short scrotum bulls (Thun et al., 1980). In calves with Bopriva® a marked delay in testicular development was obvious and at slaughter when animals were 68 weeks old paired testes weight tended to be lower in vaccinated compared to control animals. These observations are consistent with studies in the lamb (Brown et al., 1994, 1995; Clarke et al., 1998; Janett et al., 2003) where it has been shown that vaccination against GnRF in juvenile animals can lead to incomplete development of the genital tract. In this experiment the vaccinated bulls had a total sperm count of more than 3×10^9 and a sperm motility between 26 to 68 % in semen harvested from the epididymides after slaughter. These values were similar to those found in control animals indicating that the vaccinated bulls had attained spermatogenic capacity and might be judged as fertile.

Body weight gain was not influenced by immunization against GnRF which agrees with previous studies in cattle (Aissat et al., 2002; Cook et al., 2000; D'Occhio et al., 2001; Neeson and Colson, 2004) and in sheep (Brown et al., 1994; Janett et al., 2003).

5. Conclusions

This study demonstrates that immunization against GnRF with Bopriva® in bull calves at 3 and 6 weeks of age significantly inhibited the prepubertal LH rise. Furthermore, vaccination

led to a suppression of testosterone secretion for more than 22 weeks and a delay in testicular development.

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